Highlights

- SLC6 neurotransmitter:sodium symporters share a conserved architecture, yet the molecular details of their alternating access transport mechanism remain elusive.
- Studies of SLC6 membrane transporters have focused on transporter structure, rather than their environment.
- Research that includes both membrane lipids, and other environmental lipid species, could provide the keys to the molecular details of SLC6 substrate transport.

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Is protein structure enough? A review of the role of lipids in SLC6 transporter function

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25 **Abbreviations:**

26 DAT Dopamine transporter
27 DDM *n*-Dodecyl- β -D-maltopyranoside
28 DMPC 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine
29 DOPC Dipalmitoylphosphatidylcholine
30 DPPC 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
31 GABA Gamma-aminobutyric acid
32 GAT1 Type 1 gamma-aminobutyric acid transporter
33 GAT3 Type 3 gamma-aminobutyric acid transporter
34 GlyT1 Type 1 glycine transporter
35 GlyT2 Type 2 glycine transporter
36 MD Molecular dynamics
37 NET Noradrenaline transporter
38 NMR Nuclear magnetic resonance
39 PDB Protein data bank
40 PIP₂ Phosphatidylinositol 4,5-bisphosphate
41 POPC 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
42 POPE 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
43 POPG 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol
44 POPS Palmitoyl-oleoyl phosphatidylserine
45 RMSD Root-mean-square deviation
46 SERT Serotonin transporter
47 SLC Solute carrier
48 TM Transmembrane helix
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Abstract

SLC6 neurotransmitter transporters facilitate the Na^+ - and Cl^- -dependent uptake of amino acids and amino acid derivatives into cells. Disrupting transport leads to a range of neurological disorders. However, the SLC6 substrate transport mechanism is a topic of ongoing debate. Here, we review the prominent SLC6 substrate transport mechanisms through the lens of molecular dynamics simulations. SLC6 transporters are membrane proteins, yet their transport mechanism(s) have largely been studied without considering the impacts of synaptic lipid composition, or endogenous lipid modulators, on transporter structure and function. [In this review](#), we highlight the importance of studying membrane transporters in an appropriate membrane model, and present opportunities for the community to glean understanding and insight into SLC6 transporter structure and function—in particular transport mechanism(s)—when both membrane lipids and endogenous lipid modulators are considered.

Introduction to SLC6 transporters

The solute carrier (SLC) 6 family of membrane proteins perform the Na^+ -coupled symport of amino acids and amino acid derivatives into cells throughout the body. Disrupting neurotransmitter SLC6-mediated transport leads to a plethora of neurological disorders including autism, anxiety, depression, epilepsy, Parkinson's disease, schizophrenia and pain [1]. SLC6 neurotransmitter transporters are located at the synaptic cleft, with transporter-specific distributions in either the pre-synaptic neuronal membrane, or the glial cell membranes. Transporters in the presynaptic membrane remove substrate from the synaptic cleft and recycle it back into the presynaptic neuron. These are the dopamine (DAT), noradrenaline (NET), serotonin (SERT), type 2 glycine (GlyT2) and type 1 GABA transporters (GAT1). In contrast, the type 1 glycine (GlyT1) and type 3 GABA transporters (GAT3) are located in glial cell membranes, where they remove substrate from the cleft. The presynaptic and glial membranes contain distinct lipid assemblies whose role in SLC6-mediated transport is poorly understood.

While universally treating SLC6-linked neurological disorders represents a significant medical challenge, these transporters share many salient features. All SLC6 transporters characterised to date have a conserved structural architecture and substrate transport mechanism, common to other excitatory amino acid transporters in the SLC superfamily. SLC6 transporters are comprised of 600–700 amino acids, arranged into 12 transmembrane helices, with both the N- and C-termini located intracellularly (Figure 1A). Substrates are transported *via* a secondary active transport mechanism, where a molecule of substrate is co-transported with Na^+ and Cl^- down the Na^+ electrochemical gradient. The precise stoichiometry of substrate: Na^+ : Cl^- transport is specific for each type of SLC6 transporter.

Crystal structures of SLC6 transporters and their homologues indicate that substrate transport occurs via an alternating access mechanism, wherein the protein cycles through a series of conformational states while being accessible to substrates from one side of the membrane at any point in time [2,3]. In this mechanism, substrates in the synaptic cleft bind when the transporter adopts a conformation that exposes the binding site to the extracellular solution. This induces a series of conformational changes that occlude the extracellular access pathway to the substrate binding site, while opening the cytoplasmic access pathway, and allows substrate to be released intracellularly [4,5].

The precise molecular mechanisms employed by SLC6 transporters to transition between conformational states, and the role of neuronal membrane composition in this process, are difficult to characterise experimentally and thus remain largely unknown. Computational techniques such as molecular dynamics (MD) simulations can shed light on the atomic interactions governing SLC6 transporter function. In this review, we highlight recent computational advances in our understanding of SLC6 transporter function and the role of the lipid membrane in substrate transport and regulation.

SLC6 binding and transport mechanisms

Crystal structures of sodium-coupled amino acid transporters have provided clues to the binding and transport mechanism that these proteins use. Direct structural insights have only been available since 2005, when the first crystal structure of a sodium-coupled small amino acid transporter, LeuT (PDB ID 2A65) [6], was elucidated. Although LeuT is a prokaryotic transporter, its structure, combined with biochemical, mutagenesis, and homology modelling data, directed our understanding of eukaryotic SLC6 transporters. This understanding has been supplemented by the recently-solved crystal structures of eukaryotic transporters for dopamine (DAT) (PDB ID 4M48) in 2013 [7], and serotonin (SERT) (PDB ID 5I6X) in 2016 [8]. Collectively, they have provided the structural details of the substrate binding site, S1, and the location of two conserved Na⁺ binding sites, the Na1 and Na2 site. Figures 1B and 2 show the S1 site is located near the centre of the protein. It contains a polar region (formed by side chains from TM 1 and 6) that bind substrate α -amino and α -carboxylate groups, and a hydrophobic pocket (formed by side chains from TM1, TM3 and TM6) that binds hydrophobic substrate side chains. The particular amino acid composition of the S1 site, and the resultant volume and shape of the S1 cavity are believed to dictate its substrate specificity. A second putative substrate binding site, S2, has been identified in LeuT, DAT and NET [9–12]. The S2 site is located along the extracellular pathway, between the S1 site and the extracellular space [1].

While the S2 site has been implicated in binding, it is unclear whether the S2 site plays a role in the substrate transport process. Crystal structures have revealed that different drugs and detergents can bind to the S2 site of SERT [8] and LeuT [13–15]. However, there are no crystal structures of LeuT-fold transporters with substrate bound in the S2 site [16]. Recent NMR investigations of LeuT could only identify substrate binding at the S1 site [17]. Shi and co-workers proposed that the S2 site of LeuT plays an important allosteric role in the two-site substrate transport model, wherein substrate binding to S2 triggers the release of Na⁺ and substrate from the S1 site [9]. This model was computationally applied to DAT by Shan and colleagues [18] and supported by mutagenesis of DAT by Zhen and Reith [19]. Furthermore an allosteric relationship between the two sites has been shown for SERT via atomic force microscopy [20] and MD simulations [21]. Conversely, a recent investigation using a combination of MD simulations and experimental mutagenesis to examine the GlyT2 S2 site found that S2 does not influence substrate selectivity. Instead it is proposed that S2 may act as a low-affinity, non-selective loading site [22]. Taken together, these studies indicate that the role of the S2 site may differ markedly between structurally related transporters.

Structural and computational studies have revealed that SLC6 transporters shuffle between at least four states: outward-open (substrate acceptance/release, open to the extracellular space), outward-occluded (Figure 2, substrate bound, closed to the extracellular space), inward occluded (substrate bound, closed to the intracellular space) and inward-open (Figure 2, substrate acceptance/release, open to the intracellular space) [23]. However, the precise movements these transporters use to transition between the four main states are difficult to characterise using experimental techniques. Based on the available crystal structures for SLC6 transporters and computational studies, three alternating access transport models have been proposed: the rocking bundle model, the hinge model and the iris model. These models differ primarily in how they describe the movements of TM1 and TM6. The rocking bundle model, which was first devised by Forrest *et al.*, is based on mutagenesis data and

homology models of SERT in the outward-open and inward-open conformations, modelled on the LeuT structures [2]. Forrest *et al.* noticed that the LeuT crystal structure consists of two pseudo-symmetric halves, TM1–5 and TM6–10, which have inverted orientations relative to each other [2]. Computational analysis suggested that the rigid body rocking motion of TM1, 2, 6 and 7 (referred to as the four-helix bundle) within the scaffold formed by TM3–5 and TM 8–10 could provide a transition mechanism between the outward- and inward-facing conformations [2]. This conserved structural pseudosymmetry has been identified in other transporters from the SLC superfamily, including BetP, Glt_{ph} and Mhp1 [24]. Like the rocking bundle model, the hinge model also exploits the pseudosymmetry of the transporter architecture, but instead proposes that the kinked, central portions of TM1 and TM6 add subtle conformational flexibility. This precludes the rigid body motion of the four-helix bundle within the protein scaffold, that was proposed in the rocking bundle model [25]. The iris model, first proposed for the crystal structure of BetP—a bacterial sodium symporter that is structurally similar but sequence-unrelated to SLC6 neurotransmitter transporters—suggests that the pivot points for the movement of the four-helix bundle are located near the S1, Na1, and Na2 binding sites. In the iris model, the helices within the four-helix bundle are proposed to reorientate *via* a series of stepwise, anticlockwise rotations [26].

The three transport models are based on differences between the outward- and inward-facing crystallographic states. In each case, these crystallographic transitions represent static end points. Computational studies have played an integral role in identifying and characterising the dynamics associated with SLC6 transporters' outward- to inward-facing transition, and *vice versa*, as recently reviewed by Bisha and Magistrato [27]. For instance, biased simulation techniques such as steered molecular dynamics simulations have been used to pull the substrate, leucine, from its occluded binding pocket in LeuT towards either the extracellular space or the cytoplasm [9]. While carried out over relatively short timescales (< 30 ns), these simulations showed that pulling the substrate towards the cytoplasm induced hinging motions, particularly in TM1 and TM6, that the authors believed to be consistent with the hinge model [9]. A second simulation study used steered MD simulations on the outward-facing crystal structure of LeuT in conjunction with a homology model of the inward-facing LeuT (based on the vSGLT sodium/galactose transporter) to drive the conformation from an outward-facing to an inward-facing state [23]. While relatively high force constants were applied to drive the transitions, and the simulations were again conducted over short timescales, a hinging motion was noted in the intracellular halves of TM1 and TM6 of the four-helical bundle during the outward-facing to inward-facing transition. This motion was coupled to movements in the remaining helices in the four-helical bundle, and despite variations in the extent of the conformational coupling with each simulation, the authors deemed that the motions support the hinge model [23]. In fact, many other nonbiased and steered MD studies of the LeuT [28,29], and DAT crystal structures [30], and homology models of SERT [31], show increased flexibility in TM1 and TM6.

Molecular dynamics studies have proved an effective means of characterising transition state pathways between the inward- and outward-facing states, and providing the relative propensity for each state. For example, long-timescale simulations of the membrane-embedded LeuT dimer, totalling ~ 21 μ s, have been used to construct the free energy landscapes associated with the inward-facing to

outward-facing transition. The extremely long timescales used in this study also highlighted the conformational diversity and presence of low-energy “intermediate” states that may represent transition pathways from the outward-facing to inward-facing conformations [29]. Another MD study examining a membrane-embedded SERT dimer homology model showed that the type of ligand bound to the S1 site changes the propensity of SERT to transition towards an inward-facing or outward-facing state. The triplicate 60 ns simulations examined the effect of the substrate serotonin, the competitive inhibitor cocaine, and the non-competitive inhibitor noribogaine bound at the S1 site, and found that the competitive inhibitor cocaine promoted the outward-facing conformation, while serotonin and noribogaine induced transitions to the inward-facing state [32]. Similar findings have been shown for multiplex 100 ns simulations of a DAT homology model, performed with a range of inhibitors bound to the S1 site [33]. Of particular note is the conformational diversity sampled across the simulations from all three independent studies. One hallmark of MD simulations is the thermal noise inherent in the system from collective bond vibrations—molecular collisions reflective of a biological environment. While kinking and potential hinging of TM1a is observed in these simulations, collectively they show a complex ensemble of conformational changes associated with the transport cycle, which cannot be clearly delineated into any simplified model based on clear-cut morphing between crystallographic states.

Membranes and lipids in SLC6 structure and function

The role of lipids in SLC6 transporter dynamics, modulation and even structure determination is relatively unexplored. Membrane proteins are typically solubilised in detergent while being prepared for crystallographic structural determination, which removes the protein from its native bilayer environment. To date, only one SLC6 transporter, LeuT, has been crystallised in a lipid phase [34,35]. Including or removing lipids from the environment has non-trivial effects on protein structure: recent comparative studies have shown root-mean-square deviations (RMSDs) of up to 5 Å between structures of membrane proteins derived using crystallography and nuclear magnetic resonance (NMR) attributed to environmental differences [36]. In particular, NMR ensembles tend to include more kinks and a more diffuse packing arrangement in the TM helices for a given membrane protein than its corresponding crystal structure [36]. Likewise, gas phase mass spectrometry techniques show increased stability of membrane proteins in the presence of native lipids compared to their detergent solubilised counterparts [37]. These findings are supported by MD simulations of LeuT in DDM micelles, designed to mimic crystallographic conditions, which found that DDM binds to the proposed S2 substrate binding site, altering the transport stoichiometry of LeuT [38]. A follow-up computational investigation highlighted the effect of reconstitution environment: the retention of a phospholipid annulus around LeuT within the DDM micelle prevents the binding of DDM to the S2 site [12]. Therefore, if the membrane protein has been removed from its native lipid environment during crystallisation, any assessments of the impact of bilayer composition on its structural conformation, and how it influences protein function, should be interpreted with caution.

Despite a large body of research into membrane proteins, it is challenging to accurately predict their specific lipid binding sites. For instance, one recent study examined 100 high resolution membrane protein crystal structures containing bound

membrane lipids, and did not identify any common sequence motifs that could be used to predict lipid binding sites [39]. However, recent crystal structures of eukaryotic SLC6 transporters show densities for bound cholesterol, suggesting this lipid plays a structural role in transporter function. In DAT, cholesterol was resolved bound to the intracellular halves of TM1 and TM5 [7], while in SERT, cholesterol is bound to the extracellular half of TM12 [8]. In other structures, cholesterol [40,41] and the cholesterol-related compound cholesterol hemisuccinate [40] are bound to the intracellular halves of TM2, TM7 and TM11 of DAT [40,41]. While crystal structures of eukaryotic SLC6 transporters show distinct cholesterol-binding sites, as shown in yellow in Figure 1B, only three [34] of the 53 prokaryotic structures, of LeuT, show a lipid or lipid-related compound bound to the transporter. Additionally, electron densities for a range of other molecules, including sugars and detergents, decorate the surface of SLC6 transporter crystal structures, as shown in orange and maroon in Figure 1B. It is unclear whether these molecules, especially the lipids, are only required to crystallise the protein, or if they perform functional roles.

It is becoming increasingly apparent that membrane lipids play a critical role in modulating and regulating protein function. For example, embedding the homologous SLC1 transporter Glt_{ph} in a POPE bilayer allowed a third Na⁺ binding site to be identified from MD simulations [42], and the binding energy of Na⁺ at each of the specific Na⁺ binding sites to be calculated [43]. Notably, Lev and Noskov showed that when Glt_{ph} is embedded in a DPPC membrane, it is too flexible for the Na1 and Na2 sites to discriminate between monovalent ion species [44]. The regulatory effect of bilayer composition is well documented for many membrane proteins, both experimentally and in MD simulations (for example, see references [45–49]), highlighting the essential role lipids play in regulating protein function. Mass spectrometry analyses have also demonstrated that specific lipid interactions with membrane proteins stabilize structure and promote protein oligomerisation [37,50].

MD simulation techniques are powerful tools that allow the user to combine crystallographic data with model lipid bilayers to elucidate lipid-protein interactions in atomic detail. While the majority of MD simulations of SLC6 transporters have used POPE or POPC lipid bilayers, there has been little discussion of the effect of the appropriate bilayer environment on protein dynamics. For example, simulations of the bacterial LeuT transporter have been conducted in the bacterial model membrane, POPE [23,28,29], as well as the eukaryotic membrane models, POPC [51] and DMPC [52]. These differences in bilayer composition, particularly when experimentally comparing the transporter dynamics in bacterial bilayers to simulations in eukaryotic phosphatidylcholine membranes, can create inconsistencies when attempting to draw conclusive comparative results.

With the exception of the study by LeVine and colleagues [12], all of the aforementioned MD simulations of SLC6 transporters were performed with the protein embedded in a bilayer composed of a single lipid species. However, neuronal membranes are comprised of a variety of lipids [53], and with the aid of tools like the CHARMM-GUI *membrane builder* [54] and *insane* [55] it is becoming easier to computationally model bilayers that are more reflective of the native membrane, or of the experimental conditions. To date, only a small amount of research has addressed this. One study explored the effect of embedding LeuT in either a pure POPC bilayer or a 3:1 mixture of POPE and POPG, which better represents the bacterial

membrane [56]. The membrane became thinner at the protein interface, and water penetrated into the membrane in the vicinity of a membrane-facing polar residue, K288, from TM7 in both membrane types [56]. This water defect exposed hydrophobic residues from both TM7 and TM1a to the aqueous solution, creating a hydrophobic mismatch, which may influence the energetics and rate of transitions between conformational states [56,57]. In other MD simulations, which contained arseno-choline-bound BetP embedded in a 50:50 diastereoisomeric mixture of POPG lipids, a progression involving sequential Na^+ and substrate binding, dehydration, and protein coordination was observed as the transport cycle was initiated [58]. Furthermore, microsecond-timescale MD simulations of a human DAT homology model embedded in a sophisticated asymmetric bilayer—modelling a near physiological environment—have been performed. This asymmetric bilayer contained a mixture of POPC, DPPC and cholesterol in the extracellular leaflet (103:11:16 ratio) and POPE, POPC, PIP_2 , POPS and cholesterol (54:24:15:15:15 ratio) in the intracellular leaflet [59]. In this lipid environment, the *ab initio* model of the N-terminus of DAT bound spontaneously, and specifically, to the PIP_2 phospholipids. This interaction was integral to transporter function: binding to PIP_2 allowed the N-terminus to anchor to the bilayer and define the transporter's location and orientation in the bilayer. This anchoring behaviour was abolished if PIP_2 was removed from the bilayer [59]. Kinetic insight into sodium ion transport has been gained from MD simulations based on this hDAT homology model embedded in an asymmetric bilayer containing POPC, sphingomyelin and cholesterol (125:12:29 ratio) extracellularly and POPE, POPC, PIP_2 , POPS and cholesterol (92:26:18:20:24) intracellularly [60]. Notably, PIP_2 has also recently been implicated in stabilising SERT oligomer complexes experimentally [61]. Clearly, lipids can have important and relatively underappreciated impacts on the structure and function of SLC6 transporters.

Endogenous lipids as SLC6 transporter modulators

Lipids constitute part of the environment in which membrane-embedded transporters operate [62]. Endogenous lipids can act as inhibitory ligands [63] or functionally important components of the membrane that impart changes in transporter structure [64,65]. The lipid composition of the synaptic membrane is tightly regulated, to control processes including loading the correct neurotransmitters into vesicles, trafficking vesicles to the membrane, and releasing neurotransmitters during exocytosis [66]. While this process involves a range of auxiliary proteins and lipid modification enzymes, regulation of the synaptic lipid composition also modulates the activity of the SLC6 transporters within the synaptic membrane. For example, cholesterol enhances the activity of SERT [67] and DAT [68] and molecular dynamics simulations are beginning to be used to understand these processes—they have been used to propose cholesterol interaction sites in SERT [69]. Further, endogenous polyunsaturated lipids and lipid derivatives, including arachidonyl-amino acids and acyl-amino acids, have important interactions with GlyT1 and GlyT2 transporters [70–73]. Several studies have examined the interaction of anandamide with ceramides [74], cholesterol [75] and DOPC [76]; and arachidonic acid with a range of different proteins including COX-1 and -2 [77], rhodopsin [78] and 5-lipoxygenase [79], however simulation studies examining the effect of lipid derivatives on SLC6 transporters represent an under-explored area. One recent publication used crystallographic data from the cholesterol binding site of DAT, in conjunction with cysteine mutagenesis, homology modelling and MD simulations to

predict the binding site of the excitatory amino acid transporter, B⁰AT3, a homologue of LeuT and DAT [80]. Here the trafficking protein, collectrin, binds to a groove between TM5 and 7 on B⁰AT3, which corresponds to the location occupied by cholesterol in the DAT crystal structure [80], suggesting a wider role for lipid modulation in protein recognition and trafficking. Recent studies have shown that the removal of bound cholesterol accelerates conformational changes that may be associated with substrate transport in SERT and DAT [81,82]. We are only now working towards understanding how the binding of specific lipids and lipid-soluble inhibitors affects the inter-domain movements of SLC6 transporters—rearrangements required for transition to the inward-facing occluded state, and completion of the substrate transport cycle.

Future perspectives

The last 12 years have heralded unprecedented insights into SLC6 structure and function. A number of crystal structures have been solved, highlighting the overall alternating access mechanism and sparking considerable debate into the molecular interactions that govern the substrate transport process. Membrane protein crystallography and lipidomics have grown rapidly, bringing together a wealth of data that emphasise the importance of membrane composition on membrane protein function and modulation. Lipids play integral roles in biology, and their contributions to biological processes are being increasingly acknowledged. Concurrent advances in molecular dynamics simulation techniques have highlighted the molecular interactions between lipids, detergents and SLC6 proteins, providing a structural basis upon which to interpret experimental results. Furthermore, MD simulations have reinforced the stochastic nature of these membrane protein systems, which are driven by molecular fluctuations and thermal noise. However, like all techniques used to study biological systems, MD simulations are limited to the complexities of the model systems being simulated. As yet, we have not fully characterised the complexity of the synaptic membrane, or the mechanisms used by synaptic membrane lipids to modulate SLC6 transporters. Molecular dynamics simulations are ideally positioned to tease apart the complexity and dynamics of these molecular interactions, which represent a major gap in our understanding of SLC6 transporter function.

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Figure Legends

Figure 1: (A) The dopamine transporter DAT [7] (PDB ID 4M48, with transmembrane helices shown in coloured cartoon and the protein as a grey surface) with (B) an overlay of the ligands bound to all of the available SLC6 crystal structures from the protein data bank. The S1 binding site, shown in (B), readily binds substrates (magenta sticks), however there are few conserved ligand binding sites on the transporter surface. For example, the detergent molecule, β -octylglucoside (orange sticks, with red oxygen atoms) does not display a converged binding site, like other crystallographically-bound molecules (maroon sticks). In contrast, cholesterol (yellow sticks, with red oxygen atoms) binds in three distinct locations. The N-terminus of the protein is not shown in this view, but sits on the same side (intracellularly) on the protein.

Figure 2: Comparison of the LeuT crystal structures in the outward-open (PDB ID 3F3A) and inward-open (PDB ID 3TT3) state. Na^+ are shown in yellow, and the S1 site is in black. The outward-open TM1 and 6 are shown in dark red and blue, respectively, while the inward-open TM1 and 6 are pastel. The remaining inward-open helices are shown as ghosts. The membrane is shown as grey lines.

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